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Applicant:	Toyoki MORIBE et al.	Conf.:	3176
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For:	METHOD FOR TYPING OF HLA CLASS I ALLELES		

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
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Sir:

I, Toyoki Moribe, residing at 1-2-13-402 Nishi-Makami, Takatsuki, Osaka, JAPAN, do hereby declare as follows:

1. I am a citizen of JAPAN, born in 1967.

2. I graduated from Nagoya University, Japan in 1990, and received the Ph.D. degree at Osaka University, Japan in 2000. Since 1992, I have been a member of Shionogi

& Co., Ltd., Osaka, Japan, and had engaged in the works of research for genetic polymorphism, especially polymorphism of HLA genes. I am an author or co-author of 12 papers in this field.

3. I have read and understand the subject matter of U.S. Application Serial Number 09/856,662 and I am familiar with the prosecution of the application.

4. Under supervision of Dr. Toshihiko Kaneshige, I have carried out the experiments relating to the invention described below.

5. The following remarks and experimental data are submitted to show the differences between the hybridization conditions utilized in the present invention and those of Kox *et al.* (*Journal of Clinical Microbiology*, 34:2117-2120 (1996)) cited by the Examiner in the Office Action dated November 21, 2003.

6. **The Remarks and Experimental Data**

At first, I show two experiments below to explain how to decide our conditions of hybridization of the present invention.

Experiment 1. Comparison of hybridization temperature

Methods

Amplification of the region containing the exon 2, the intron 2 and the exon 3 of the HLA-B allele from genomic DNA which was isolated from the sample with the HLA-B allele type of B*0702, B*0702, was performed by the PCR method using biotinylated 5BIN1-TA, 5BIN1-CG and 3BIN3-37 for a primer pair. The amino-modified SSO probes, BL77, BL527A, BL1 and BL226G, were covalently immobilized onto carboxylate-modified microtiter plates by incubating over night at room temperature with 0.1 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl) in 10 mM MES buffer (pH 5.4). Each well of the SSO probe-immobilized microtiter plates was pre-hybridized with 100 μl of hybridization buffer (GMC buffer) (0.25 M di-sodium hydrogenphosphate, 1 mM EDTA, 7% SDS, 1% BSA and 20% formamide (pH 7.0)) for 10 min at 37 °C in an air incubator or room temperature. During pre-hybridization, 32 μl of each biotinylated PCR amplified product was denatured with an equivalent volume of 0.4 N NaOH solution for 5 min at room temperature, followed by the addition of 100 μl of GMC buffer. The pre-hybridization buffer was discarded from the microtiter plates, and 100 μl of hybridization buffer containing denatured PCR product was added to each well. The plates were sealed and incubated for 1 hour at 37 °C in an air incubator or room temperature. Following hybridization, the wells were washed 5 times with 2 x SSC (0.3 M sodium chloride and 30

mM tri-sodium citrate) at room temperature. To detect biotinylated PCR products which hybridized with SSO probes, 100 μl of peroxidase-conjugated streptavidine solution, diluted to 1/2000) in T-TBS buffer (20 mM Tris-HCl, 0.5 M sodium chloride and 0.5% Tween 20 (pH 7.6)), was added to each well. The plates were then incubated for 15 min at 37 °C in an air incubator. The wells were washed 5 times with T-TBS buffer at room temperature. To each well, 100 μl of TMB (3,3',5,5'-tetramethyl benzidine) solution as a substrate was added, and the plates were incubated for the colorimetric reaction for 30 min at 37 °C in an air incubator. After color development, the reaction was stopped with the addition of 100 μl of 1% SDS and the absorbance was measured at 650 nm.

Results

As shown in Table 1, the absorbance values to positive probes at 37 °C and room temperature were almost same and reached a plateau. However, the absorbance values to negative probes were very high at room temperature due to non-specific hybridization to negative probes, while those were very low and less than 0.5 at 37 °C. Therefore, only at 37 °C, the positive and negative signals were successfully defined.

In conclusion, hybridization temperature which was suitable to HLA class I DNA typing by our method, was 37 °C, but not room temperature.

Experiment 2. Optimal concentration of formamide in hybridization buffer

Methods

Amplification of the region containing the exon 2, the intron 2 and the exon 3 of the HLA-B alleles from genomic DNAs which were isolated from two samples with the HLA-B allele type of B*4002, B*4002 and of B*5101, B*5101, respectively, was performed by the PCR method using biotinylated 5BIN1-TA, 5BIN1-CG and 3BIN3-37 for a primer pair. The amino-modified SSO probe, BL1, was covalently immobilized onto carboxylate-modified microtiter plates by incubating over night at room temperature with 0.1 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl) in 10 mM MES buffer (pH 5.4). Each well of the SSO probe-immobilized microtiter plates was pre-hybridized with 100 μl of hybridization buffer (GMC buffer) (0.25 M di-sodium hydrogenphosphate, 1 mM EDTA, 7% SDS and 1% BSA (pH 7.0)) containing 0, 20, or 40% formamide for 10 min at 37 °C in an air incubator. During pre-hybridization, 4 μl of each biotinylated PCR amplified product was denatured with an equivalent volume of 0.4 N NaOH solution for 5 min at room temperature, followed by the addition of 100 μl of GMC buffer containing 0, 20, or 40% formamide. The pre-hybridization buffer was discarded from the microtiter plates, and 100 μl of hybridization buffer containing denatured PCR product was added to each well. The plates were sealed and incubated for 1 hour at 37 °C in an air incubator. Following hybridization, the wells were washed 5 times with 2 x SSC (0.3 M sodium chloride and 30

mM tri-sodium citrate) at room temperature. To detect biotinylated PCR products which hybridized with the SSO probe, 100 µl of alkaline phosphatase-conjugated streptavidine solution, diluted to 1/1000 in T-TBS buffer (20 mM Tris-HCl, 0.5 M sodium chloride and 0.5% Tween 20 (pH 7.6)), was added to each well. The plates were then incubated for 45 min at 37 °C in an air incubator. The wells were washed 5 times with T-TBS buffer at room temperature. To each well, 100 µl of DEA solution (10% diethanolamine and 1 mM magnesium chloride (pH 9.8)) containing 4 mg/ml PNPP (p-nitrophenylphosphate) as a substrate was added, and the plates were incubated for the colorimetric reaction for 30 min at 37 °C in an air incubator. After color development, the reaction was stopped with the addition of 25 µl of 0.5 M EDTA and the signal was judged visually by the naked eye.

Results

As shown in Table 2, only at 20% formamide concentration, the signals to positive and negative samples were clearly defined. At 40% formamide concentration, the signal to positive sample was hardly visible to the naked eye and not discriminated from the signal to negative sample because of high stringency of hybridization condition. The signal to negative sample was very strong and comparable to the signal to positive sample due to non-specific hybridization to the negative sample at 0% formamide concentration.

In conclusion, the optimal concentration of formamide was 20% for HLA class I

DNA typing by our method.

Our hybridization conditions that the temperature is from 32 to 42 °C and the formamide concentration is from 5 to 30 % were determined based on the results of the above experiments. The positive and negative signals were not defined by hybridization performed at room temperature or 40 % formamide concentration.

On the other hand, Kox *et al.* discloses a microwell hybridization assay including hybridization reaction in the presence of 50 % formamide at room temperature (page 2118, column 1, line 35 and 39). Kox *et al.* examined whether there is any PCR products by a hybridization method. Their conditions can not be used for our invention based on the results of the above experiments. We need a hybridization method which can recognize the difference of only single nucleotide, and our hybridization conditions must be controlled more strictly than those of Kox *et al.* However, Kox *et al.* failed to disclose the hybridization conditions in our invention.

I hereby declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section

1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 29 day of January, 2004.

Toyoki Moribe

Toyoki Moribe, Ph.D.

Table 1. Comparison of the absorbance values in two conditions of hybridization temperature using the sample with the HLA-B allele type of B*0702, B*0702

SSO probes	Hybridization temperature	
	37 °C	Room temperature
BL77 (Positive probe)	2.404	2.384
BL527A (Positive probe)	2.575	2.177
BL1 (Negative probe)	0.311	1.485
BL226G (Negative probe)	0.223	2.072

Table 2. Signal judgment by the naked eye for reactivity of the SSO Probe, BL1, at different formamide concentrations

HLA-B allele type	Formamide concentration		
	0%	20%	40%
B*4002, B*4002 (Positive sample)	+++	+++	-
B*5101, B*5101 (Negative sample)	+++	-	-

+++ : strong signal - : no signal